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INFLUENCE OF FK 506 (TACROLIMUS) ON CIRCULATING CD4$^+$ T CELLS EXPRESSING CD25 AND CD45RA ANTIGENS IN 19 PATIENTS WITH CHRONIC PROGRESSIVE MULTIPLE SCLEROSIS PARTICIPATING IN AN OPEN LABEL DRUG SAFETY TRIAL

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We have taken the opportunity of a clinical trial of the potential efficacy and safety of FK 506 (tacrolimus) in chronic progressive multiple sclerosis (MS) to examine the influence of this potent new immunosuppressant on circulating T-lymphocytes in an otherwise healthy non-transplant population. Peripheral blood levels of subsets of CD4$^+$ T lymphocytes expressing the activation molecule interleukin-2 receptor (p55 α chain; CD25) or the CD45RA isoform were determined sequentially in 19 patients that were treated continuously with oral FK 506 (starting dose 0.15 mg/kg/day) for 12 months. No significant change in the proportion of circulating CD25$^+$ CD4$^+$ cells was observed over the study period in which the mean trough plasma FK 506 level rose from 0.3 ± 0.2 to 0.5 ± 0.4 ng/ml. There was also no significant effect of FK 506 on the percentage of CD45RA$^+$CD4$^+$ cells in the peripheral blood at 12 months compared with pretreatment values. Analysis of a subgroup of 7 patients, who showed a sustained reduction in CD25$^+$ CD4$^+$ cells and a reciprocal increase in CD45RA$^+$ CD4$^+$ cells for at least 6 months after start of treatment, did not reveal any difference in disability at one year compared with the treatment group as a whole. The side effects of FK 506 were mild and the overall degree of disability estimated by the mean Kurtzke expanded disability status scale (EDSS) score or the ambulation index did not deteriorate significantly in the 19 patients studied over the 12 months of FK 506 administration.

KEY WORDS: Multiple sclerosis, FK 506, immunosuppression, T cells.

ABBREVIATIONS: AI: ambulation index; CNS, central nervous system; EDSS, expanded disability status scale; mAb, monoclonal antibody; MHC, major histocompatibility complex; MNC, mononuclear cells; MS, multiple sclerosis.

INTRODUCTION

The macrolide antibiotic FK 506 (tacrolimus) is a powerful new T-cell-directed immunosuppressive agent[1,2] with a similar but considerably more potent anti-lymphocytic activity compared to cyclosporin A (CsA)[3,4]. It suppresses selectively, the transcription of interleukin-2 (IL-2) and other cytokine genes in CD4$^+$ T-cell[5], and inhibits the induction of T-cell activation marker expression (e.g. major histocompatibility complex [MHC] class II antigen and IL-2 receptor [IL-2R]) both *in vitro*[6] and following systemic immunization *in vivo*[7]. FK 506 has been shown to prevent and reverse human organ transplant rejection[8] and has recently been approved for the therapy of liver allograft rejection. The immunosuppressive efficacy and safety of FK 506 have also been demonstrated in certain organ-specific and systemic autoimmune diseases, both in animals and in preliminary clinical trials[9–13]. There have however, been no detailed reports of the influence of FK 506 on circulating T cells in non-transplant patients receiving the drug. We have taken the opportunity of a clinical trial of the potential efficacy and

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safety of FK 506 in chronic progressive multiple sclerosis (MS) to investigate the influence of the drug on circulating T cells in a non-transplant population that was otherwise in good health.

It is widely believed that MS is a chronic autoimmune disease of the central nervous system (CNS), that is mediated by T-lymphocytes that recognize an as yet unidentified autoantigen[14]. MS is associated with abnormalities in the regulation of immune reactivity, including changes in T-cell subset distribution that resemble those seen in other autoimmune diseases[14–16]. Recently, significant changes in the proportion of $CD4^+$ cells expressing the CD45 isoforms CD45RA or CD45RO/CD29, that represent different maturational stages of T cells[17] have been reported in several autoimmune[18,19] or immunodeficiency disorders[20]. It has been reported that, in progressive MS, there is a selective loss of circulating $CD4^+$ T-cells expressing CD45RA (2H4), — a marker of "suppressor-inducer" (naive) cells[18,21]. Therapies which may reverse this imbalance in regulatory T cell populations could prove valuable in the treatment of the disease. We have examined the influence of FK 506 on peripheral blood $CD4^+$ cells expressing the IL-2Rα chain (CD25) and the CD45RA isoform in 19 patients with chronic progressive MS to ascertain whether any observed changes might be related to and/or predictive of response to therapy with this important new anti-lymphocytic agent.

MATERIALS AND METHODS

Subjects

The 19 patients studied were part of a randomized, drug concentration-controlled pilot study of the potential efficacy and safety of FK 506 in chronic progressive MS. The trial was approved by the University of Pittsburgh Medical Center (UPMC) Institutional Review Board and by the US Food and Drug Administration. Seven male and 12 female patients, age range 25–67 years (mean 43.4 ± 10.4 years) with clinically definite MS of at least one years duration and from whom a pretreatment and sequential, 3-monthly blood samples were available were studied during outpatient visits to the UPMC. Progressive MS with accumulating disability is a clinical pattern characterized by an increase in neurological impairment and disability over time. This definition required that each patient maintained a progressive pattern with accumulating disability in the six months before entering the study. Subjects with exacerbating and remitting disease without progression were excluded. In order to be eligible for the study, patients were at a level of disability as defined by the Kurtzke Expanded Disability Status Scale (EDSS)[22] of grade 3.0 to 7.0 inclusive. The pretreatment Kurtzke scores ranged from 3.0–7.0 (mean 5.8 ± 1.1) and the ambulation index[23] (AI) range (see below) was 2.0–7.0 (mean 4.5 ± 1.3). No patients had received steroids and ACTH for at least one month, or immunosuppressive drugs for at least 3 months at the time of entry and initial blood sampling. All patients were required to be co-operative and in otherwise good general health. In particular, subjects with a history of alcoholism, hepatitic disease, renal dysfunction, cardiac disorders, pulmonary or other illnesses were excluded. Normal healthy adult controls were laboratory volunteers of both sexes with a similar age range to the MS patients.

Clinical assessment

At entry, all patients underwent an extensive physical and neurological examination and a number of hematological and blood chemical studies. Magnetic resonance imaging (MRI) was performed to confirm the diagnosis of MS. General medical examination included measurements of blood pressure, temperature, respiratory rate, weight, chest x-ray and electrocardiogram. Standard laboratory testing included the following: blood sugar, electrolytes, blood urea, serum creatinine and calcium, complete blood count, coagulation profiles, liver screen, serum amylase and urinalysis.

Neurological evaluation was conducted by neurologists trained in the application of the specific scoring systems employed during the study. Two measurement systems were used, namely the Kurtzke (= EDSS) score[22] and the ambulation index (AI).[23] These scoring systems are commonly used in clinical trials involving MS patients.[24] The Kurtzke score involves the assessment of a variety of functional systems covering the major areas examined during clinical evaluation of the nervous system. Each of these functions is graded on a scale from 0 (normal) to maximum impairment (grade 6). Data from evaluation of individual functional systems is then incorporated into an EDSS score which ranges from grade 0 (normal neurological examination) to grade 10 (death from MS). The second clinical performance indicator used was the AI. This test focuses on the ability of the subject to ambulate: the grades range from 0 to 10, grade 0 being an asymptomatic, fully active individual while grade 10 applies to a totally bedridden patient. Intervening grades are standardized to reflect progressive levels of impairment.

Patients were seen initially at entry, then at weekly intervals for one month, thereafter at monthly intervals for a period of three months, then every subsequent three months. Laboratory testing, a general medical evaluation, and neurologic function were assessed at each visit.

FK 506 administration and plasma levels

All of the patients studied received continuous FK 506 treatment for 12 months. After randomization into 1 of 3 plasma concentration ranges (low, 0.1–0.3 ng/ml; medium, 0.3–0.7 ng/ml or high, 0.7–1.3 ng/ml) FK 506 (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan) was started at a daily oral dose of 0.15mg/kg, which represented half that given initially to organ transplant patients. This lower dose was selected to reduce the risk of side effects in a non-life-threatening condition in which there was no guarantee of therapeutic efficacy. Once therapy commenced, doses were adjusted using a dosing algorithm as described previously[25]. Dose reduction was allowed in the event of toxic side effects. The mean and median daily dose of FK 506 and early morning plasma trough levels of the drug were determined by enzyme-linked immunosorbent assay[26] at 0.3,6,9 and 12 months after the start of FK 506 administration (Table 1).

Immunophenotypic analysis of blood lymphocytes

Anticoagulated peripheral venous blood samples were obtained before the start of treatment (day 0) and at 7 days, 1,3,6,9 and 12 months thereafter. Mononuclear cells (MNC) were isolated from the samples by centrifugation over Histopaque (Sigma, St. Louis, MO) and stained using standard procedures, as previously described[6]. Mouse anti-human monocloncal antibodies (mAbs) used in one or two-color flow cytometric analysis of patients' blood MNC or of those obtained from healthy adult controls included phycoerythrin (PE)-conjugated anti-CD4, PE-conjugated anti-CD8, and FITC-conjugated anti-CD25 (IL-2R, p55 α chain) and were diluted 1/10 in Hanks' balanced salt solution (HBSS) with 1% bovine serum albumin (BSA) and 0.1% NaN_3. PE-conjugated mouse IgG and FITC-conjugated mouse IgG were used as isotopic controls. These antibodies were purchased from Dako, Carpinteria, CA. To determine the proportion of $CD45RA^+$ cells within the $CD4^+$ cell population, a kit containing FITC-conjugated T4 and RD1-conjugated anti-2H4 was purchased from Coulter Immunology, Hialeah, FL. The mAbs were diluted 1/10 in the HBSS/1% BSA/0.1% NaN_3 buffer. Cells isolated from blood samples were incubated with diluted antibodies for 30 min at 4°C. After incubation, the cells were washed twice with buffer and then fixed in 1% paraformaldehyde until analysis by flow cytometry. Five thousand gated events were counted, using a FACSTAR® flow cytometer (Becton-Dickinson, San Jose, CA) and the results were expressed as percentage positive cells.

Statistical analyses

The rate of change in percentage of positively staining cells was determined for each patient using simple logistic regression[27]. The Wilcoxon Sign Rank test, a non-parametric equivalent to the paired "t"-test was used to compare pre-and post (12 months) FK 506 values, as well as pre- and post-treatment indices of disease activity. The Wilcoxon Rank Sum test was used to compare pre-FK 506 values and those obtained from normal volunteers.

RESULTS

Daily dose and plasma level of FK 506

The mean and median total daily dose and 12-hr trough plasma levels of FK 506 in the 19 MS patients taking the drug continuously for 12 months are shown in Table 1. Whilst the daily FK 506 dose rose during this period, the range became more restricted. Although the median plasma concentration of FK 506 remained unchanged throughout the study, there was

Table 1 Daily dose and plasma level of FK 506 in MS patients

	Months after start of treatment (n = 19)				
FK 506	0	3	6	9	12
Dose (mg)					
mean (SD)	5.5(4.2)	5.8(2.6)	5.9(1.9)	6.7(3.0)	7.9(3.2)
median	4.0	6.0	6.0	7.0	8.0
range	2.0–18.0	2.0–10.0	2.0–10.0	2.0–12.0	2.0–14.0
Plasma trough level (ng/ml)					
mean (SD)	–	0.3(0.2)	0.4(0.3)	0.4(0.3)	0.5(0.4)
median	–	0.3	0.3	0.3	0.3
range	–	0.1–0.9	0.1–1.0	0.1–1.1	0.1–1.8

Table 2 Biochemical indices of renal impairment in FK 506-treated MS patients

Parameter	Baseline	Months after start of treatment (n = 19)				
		3	6	9	12	p-value†
Serum creatinine (mg/dL)	0.85 ± 0.22	0.94 ± 0.16	0.95 ± 0.20	0.92 ± 0.20	0.98 ± 0.26	0.037
BUN* (mg/dL)	12.9 ± 3.7	13.8 ± 5.3	14.0 ± 3.2	15.5 ± 4.7	17.3 ± 5.3	0.008

† 12-month compared with baseline value.
*blood urea nitrogen.

an increase in the mean concentration from 0.3 ± 0.2 to 0.5 ± 0.4 ng/ml.

Influence of FK 506 on renal function

Nephrotoxicity is one of the well-known potential side effects of FK 506[28]. As shown in Table 2, the mean serum creatinine and blood urea nitrogen (BUN) levels rose to 1.2 and 1.3 times baseline respectively, over the 12 months of study.

Patient Disability

The mean Kurtzke EDSS score and ambulation index in the 19 MS patients over the 12-month study period are shown in Table 3. The mean change in Kurtzke score at 1 year was + 0.06 ± 0.73 (n = 19). Overall, there was essentially no change in either of these clinical indices of patient disability during the study. MRI data were available for the 19 patients at entry and at 1 year. The overall impression was that there was no quantifiable change in lesion load.

Change in Kurtzke score or AI in relation to individual plasma FK 506 levels

As shown in Fig. 1, there was a tendency to higher mean plasma FK 506 concentrations in those patients that showed improvement in AI (increase of 1 relative to baseline) or Kurtzke score (decrease of 0.5 relative to baseline)[24] at 12 months.

Adverse effects

The ten most common adverse events recorded in the FK 506-treated patients and their prevalence of occurrence are shown in Table 4. None of these events required specific treatment or necessitated withdrawal of patients from the study.

Expression of CD25 and CD45RA on peripheral blood CD4⁺ cells

To determine the influence of continuous systemic FK 506 administration on the percentage of activated ($CD25^+$) and immunoregulatory ($CD45RA^+$) $CD4^+$ lymphocytes, two-color flow cytometric analysis of peripheral blood MNC was performed before the start of treatment and at intervals thereafter, up to 1 year. Table 5 shows that before treatment, $CD4^+$ cells and the CD4: CD8 ratio were elevated significantly above normal, unlike $CD8^+$ cells, which were significantly decreased in the 19 patients studied. The proportion of either $CD4^+$ or $CD8^+$ cells bearing the CD25 marker was elevated above normal, as was the proportion of $CD45RA^+$ $CD4^+$ cells. When pre- and post-treatment (12-month) values were compared, there was no effect of FK 506 on any of the parameters. Values obtained for $CD25^+$ and $CD45RA^+$ $CD4^+$ cells during the 12-month study period are shown in Figure 2. There was essentially no change in the median percentages of $CD25^+$ $CD4^+$ cells compared with the pretreatment level during the 12 months of treatment. The proportion of $CD45RA^+$ $CD4^+$ cells also did not change significantly throughout the study, although the median was 25% below the pretreatment value at 12 months.

Table 3 Kurtzke (EDSS) and ambulation index (AI) scores

Score	Months after start of FK 506 treatment (n = 19)				
	0	3	6	9	12
Kurtzke (EDSS) (mean ± SD)	5.9 ± 1.1	5.8 ± 1.2	5.8 ± 1.4	5.6 ± 1.3	5.8 ± 1.3
AI (mean ± SD)	4.5 ± 1.3	4.5 ± 1.6	4.5 ± 1.5	4.3 ± 1.4	4.6 ± 1.5

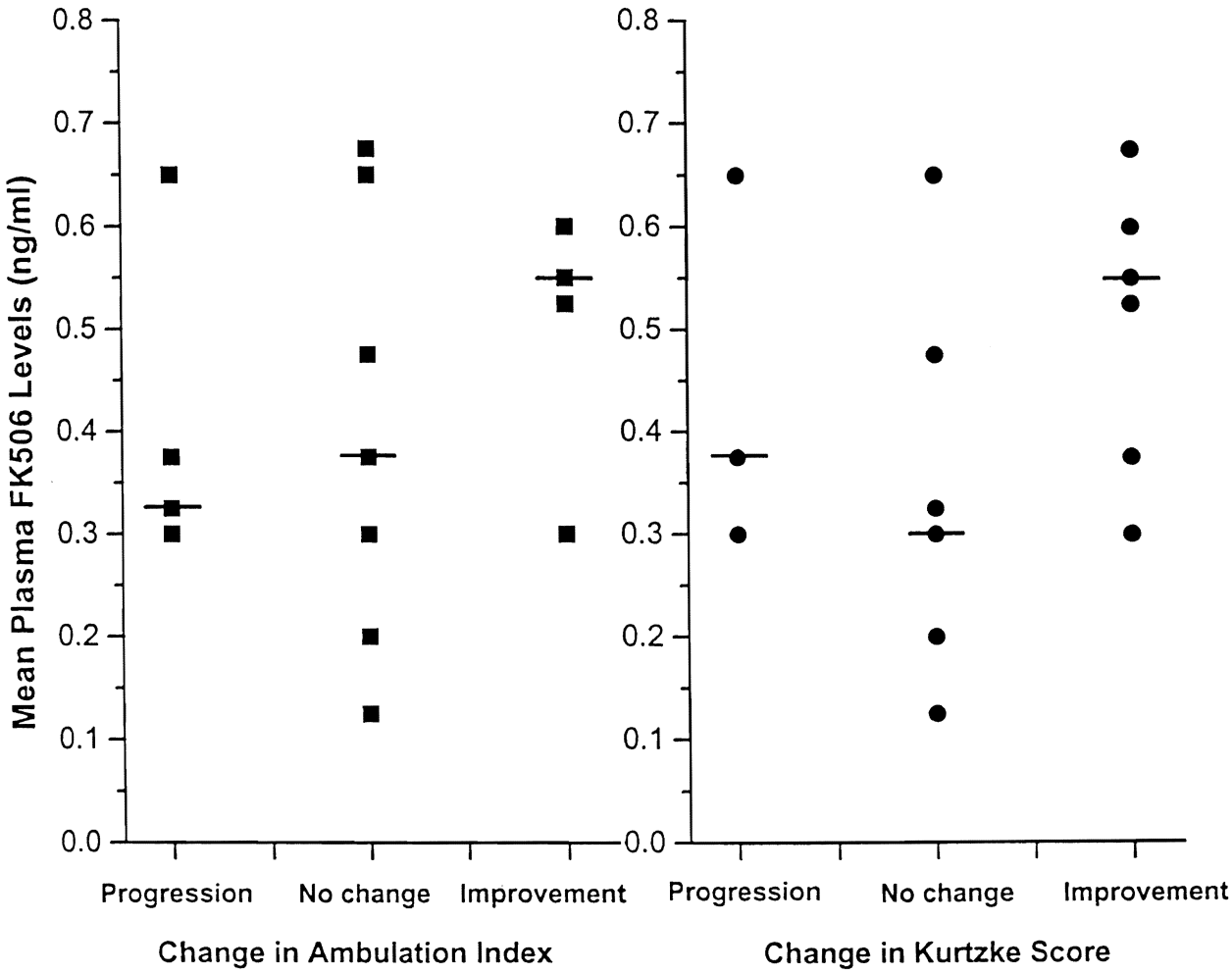

Figure 1 Change in the ambulation index and Kurtzke (EDSS) score in relation to mean individual plasma FK 506 concentration (over the 12-month study period) for the 19 chronic progressive MS patients studied.

Table 4 The ten most common adverse reactions in FK 506-treated MS patients (n = 19)

Effect	Prevalence (%)
Fatigue*	44.4
Decreased Appetite*	38.9
Tremors	38.9
Headache	33.3
Light Sensitivity	29.4
Blurred Vision*	27.8
Insomnia	22.2
Abdominal Pain	22.2
Hair Loss	22.2
Hypertension†	21.1

*may be an overestimate because of difficulty in differentiating disease-related symptomatology from drug-induced effects.
†incidence of patients on anti-hypertensive medication at 1 year.

Patterns of change in CD45RA$^+$ and CD25$^+$ CD4$^+$ cells in individual MS patients

In several patients, an increase in CD45RA$^+$ ("suppressor-inducer") CD4$^+$ cells following start of FK 506, was accompanied by a concomitant decrease in CD25$^+$ (activated) CD4$^+$ cells. It was of interest to determine whether these changes were related to grade of disability. Sequential determinations were therefore made on subjects with starting values of CD45RA$^+$ CD4$^+$ cells $\geq$ 10% below the normal mean *and* of CD25$^+$ CD4$^+$ cells > 1 SD above the normal mean. Patients in which an increase in CD45RA$^+$ CD4$^+$ cells *and* a decrease in CD25$^+$ CD4$^+$ cells was sustained for at least 2 occasions post treatment (7 subjects) were examined further. Figure 3 shows that in this sub-group of FK 506 treated patients, there was no correlation between these laboratory indices of T cell function and the clinical assessment of patient disability over the follow-up period.

Table 5 Expression of CD25 and CD45RA on peripheral blood T cells of 19 MS patients treated with FK 506

Antigen(s)	Normal (n = 12)	FK 506-treated Pre (n = 19)	FK 506-treated Post (12 months) (n = 19)	"p"-value a	"p"-value b
	a		b		
$CD4^+$	37.8 ± 4.5	47.8 ± 9.1	45.2 ± 10.2	< 0.001	NS
$CD8^+$	34.8 ± 6.4	24.5 ± 5.4	24.6 ± 8.3	< 0.001	NS
CD4 : CD8	1.1 ± 0.2	2.1 ± 0.8	2.1 ± 1.0	< 0.001	NS
$CD25^+$ $CD4^+$*	9.3 ± 2.1	11.6 ± 6.6	12.6 ± 7.4	NS	NS
$CD25^+$ $CD8^+$	0.8 ± 0.6	2.5 ± 2.5	3.6 ± 3.8	0.001	NS
$CD45RA^+$ $CD4^+$	17.2 ± 7.1	24.1 ± 10.4	20.5 ± 13.4	NS	NS

*Determined by two-color immunofluorescence staining; value is % $CD4^+$ or $CD8^+$ cells, as appropriate.

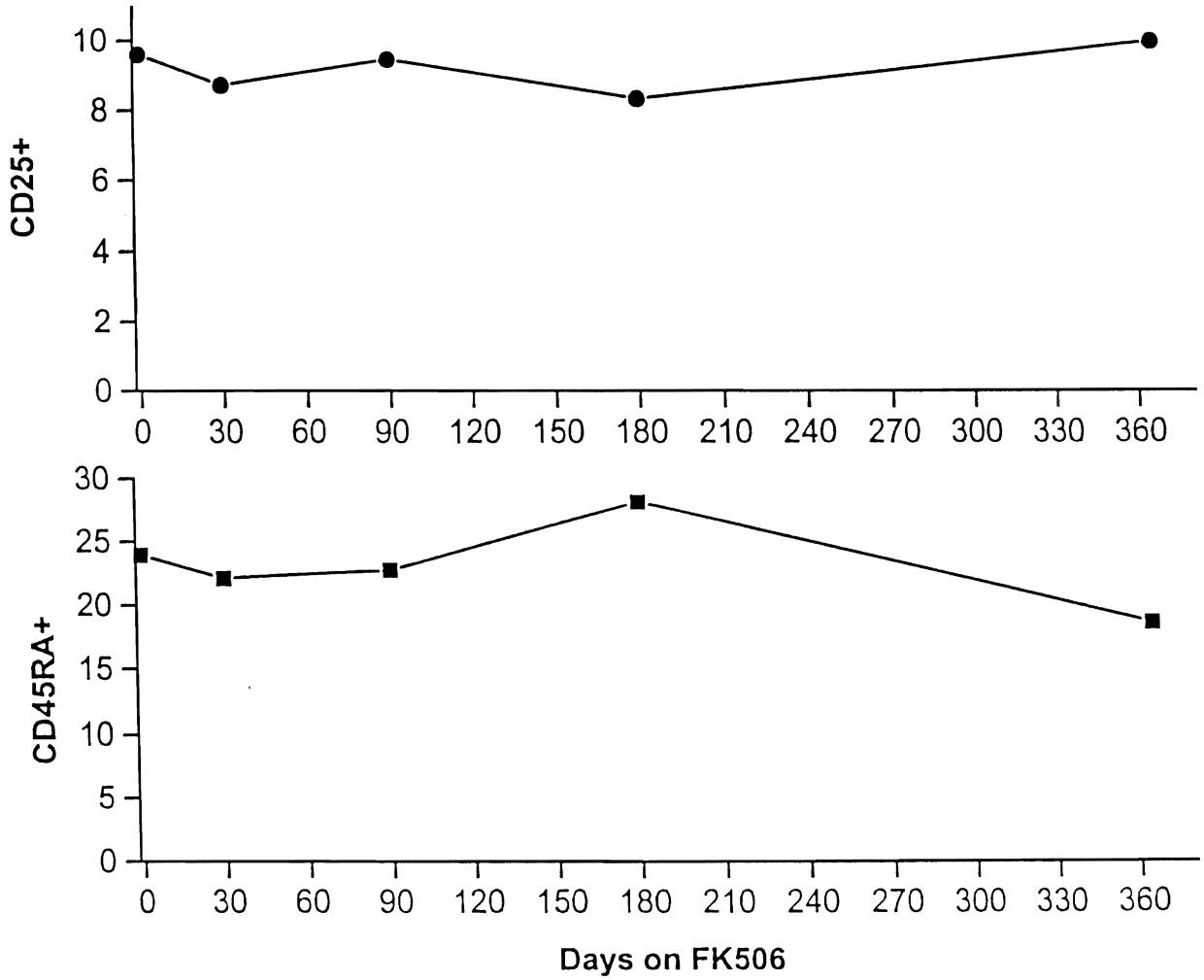

Figure 2 Percentages of $CD25^+$ $CD4^+$ and $CD45RA^+$ $CD4^+$ cells (median values) in peripheral blood of 19 chronic progressive MS patients at various times after the start of FK 506 immunosuppressive therapy.

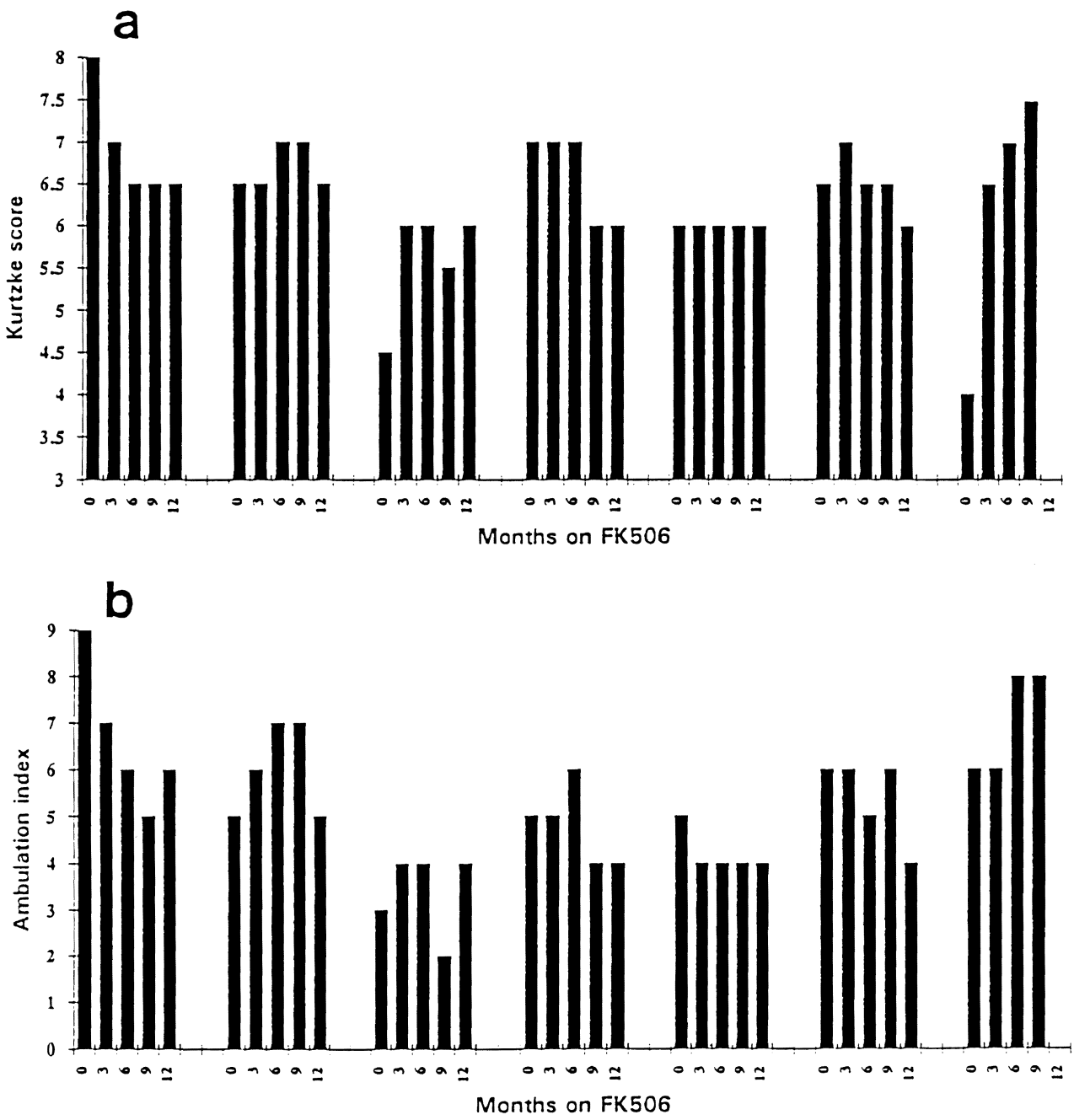

Figure 3 Disability assessment (Kurtzke EDSS score and ambulation index) and corresponding percentages of $CD25^+$ $CD4^+$ and $CD45RA^-$ $CD4^+$ cells in blood of 7 patients with chronic progressive MS at various times after start of FK 506 treatment. This subgroup of patients had a high initial (day 0; pre FK 506) level of $CD25^+$ $CD4^+$ cells and a concomitant low level of $CD45RA^-$ $CD4^+$ cells compared with normal subjects and showed a decrease and increase respectively in these parameters following instigation of immunosuppressive therapy. For definition of "low" and "high" values see Results section.

DISCUSSION

This investigation was designed primarily to evaluate the potential efficacy and toxic side effects of FK 506 in patients with pre-existing neurological disease. Such a study is of importance as there is an indication that FK 506 may, under certain circumstances, be toxic to the nervous system[29]. The study was not designed primarily to determine the effect of FK 506 on the course of MS and hence a traditional, double-blind, placebo-controlled investigation was not carried out. Important clinical information can nonetheless be de-

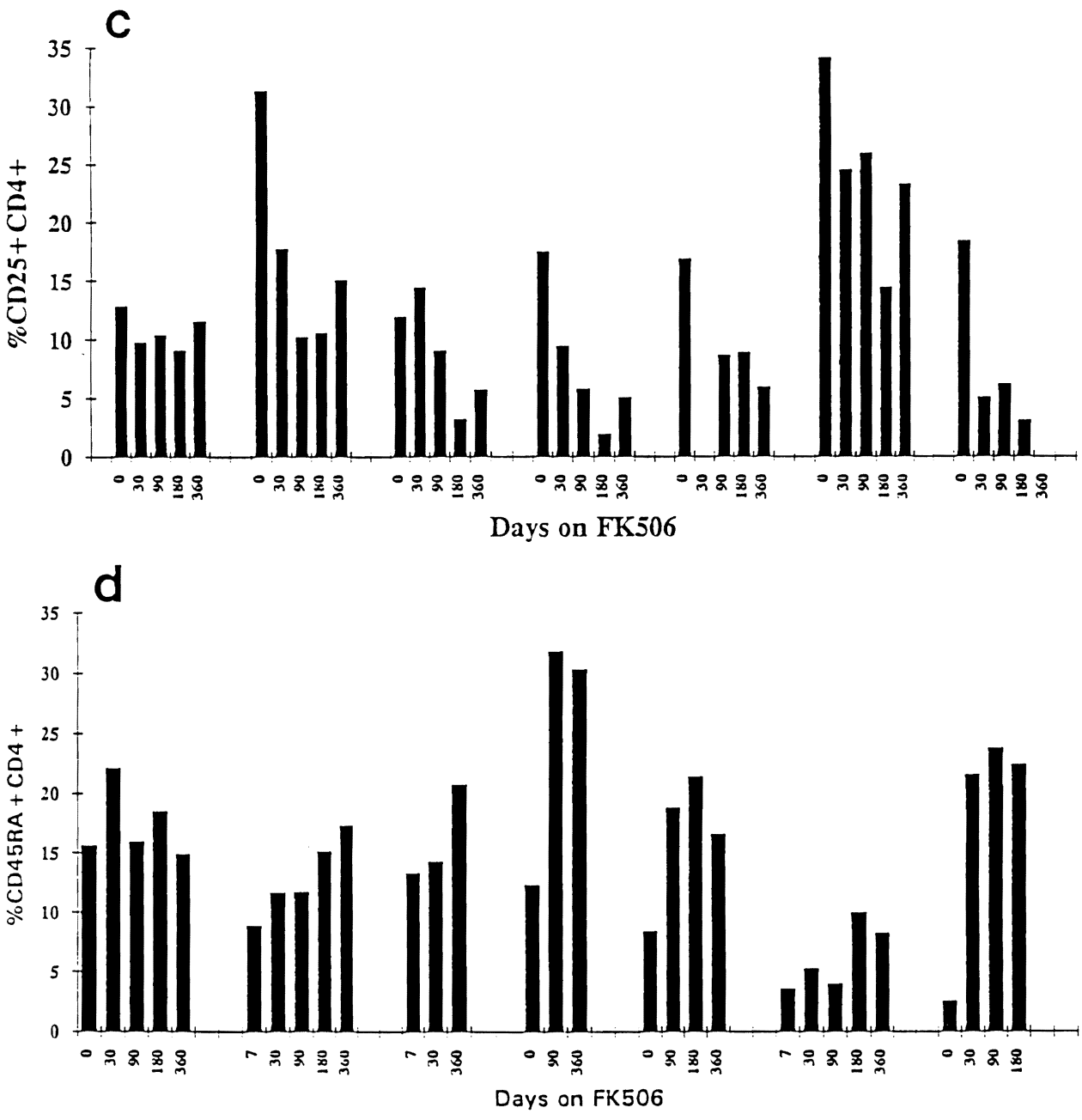

Figure 3 (*continued*).

rived from a study of this nature[30]. Further information will be forthcoming once the full cohort of MS patients (105) has completed the two-year study. The 19 patients selected for the immunological analyses represented a minor proportion of the total patient group. While no statistical information can be drawn from the clinical data, the results indicate, nonetheless, that in these patients, at least clinical stability was evident throughout the course of treatment. Patients were chosen on the basis of chronic disease progression and all had deteriorated by at least one score on the Kurtzke EDSS during the year before FK 506 administration. The mean change in Kurtzke score in the 19 FK 506-treated patients after 12 months was + 0.06 ± 0.73. Thus, the results show a trend to stabilization of the MS while on FK 506 treatment. There was also a tendency to a higher mean plasma FK 506 level in those patients that showed improvement in Kurtzke score (decrease of 0.5 relative to baseline). The side effects of the drug were mild in the 19 patients, and in

no case were they deemed severe enough to warrant discontinuation of the medication.

The present study focuses on the influence of FK 506 on circulating T cells in chronic progressive MS. Previous studies have shown that activated T-cells are increased in the peripheral blood of patients with this condition[31]. Moreover, T-cells expressing cell surface activation molecules, such as IL-2R and MHC class II antigens, can be identified in lesional tissue within the CNS of MS patients[32]. In the present investigation, significantly increased incidences both in circulating total $CD4^+$ cells and in the CD4 : CD8 ratio were observed in the 19 progressive MS patients compared with healthy adult control subjects. Increases both in activated $CD25^+$ $CD4^+$ and $CD25^+$ $CD8^+$ T cells (especially) were also found compared with healthy age- and sex-matched individuals. These findings suggested impaired immune regulation, consistent with previous reports of T cell populations in this disease. In contrast with an earlier study[21], and our own previous observations on a smaller group of these patients[33], the mean proportion of immunoregulatory $CD45RA^+$ $CD4^+$ cells in peripheral blood was not decreased significantly pretreatment compared with healthy subjects. There was however, considerable inter-individual variation in this parameter. Several patients showed a decrease (> 10%) in this subset of $CD4^+$ cells that has been correlated positively with T-cell suppression and with inhibition of T- and B-cell clones reactive with elements of the CNS[21].

Treatment with FK 506, a potent inhibitor of $CD4^+$ T-cell activation in response to stimulation via the CD3/T cell receptor pathway, did not affect significantly the proportion of either $CD25^+$ or $CD45RA^+$ $CD4^+$ cells over a 12-month period. Moreover, no relationship could be demonstrated between an increase in $CD45RA^+$ $CD4^+$ cells together with a concomitant decrease in $CD25^+$ $CD4^+$ cells and the patients' disability status in response to FK 506. Notably, at the FK 506 doses used and at the plasma levels achieved (which are anti-lymphocytic *in vitro*,[1,2,5,6] and similar to or lower than those reported previously in patients with uveitis[34], or psoriasis[9]) there was no significant overall change in the clinical status of the MS patients.

While this study has examined the influence of FK 506 on non-specific indices of peripheral blood $CD4^+$ cell activation and function, the possibility that this new anti-T cell agent might exert effects on the function of specific, disease-associated T cells both within the blood and more significantly, within the CNS, cannot be excluded. Indeed, local inhibition of inflammatory events within the skin have recently been reported in FK 506-treated psoriasis patients.[11,35,36] Although FK 506 has not been detected in the cerebrospinal fluid of several organ transplant patients with very high plasma drug levels exhibiting toxic manifestations of FK 506 (R. Venkataramanan, Dept. of Pharmacy, University of Pittsburgh; personal communication) studies on T-cell populations within lesional tissue of FK 506-treated patients with pre-existing neurological disease would be very informative.

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